

## Extracellular Polysaccharide Is Not Responsible for Aluminum Tolerance of *Rhizobium leguminosarum* bv. Phaseoli CIAT899

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**Strain UHM-5, a pSym<sup>-</sup> Exo<sup>-</sup> derivative of the aluminum-tolerant *Rhizobium leguminosarum* bv. phaseoli strain CIAT899, was equally tolerant of aluminum (Al) as the parental culture. Dialyzed culture supernatants of the wild-type cells grown in YEM broth (10<sup>9</sup> cells ml<sup>-1</sup>) contained 185 µg of glucose equivalents ml<sup>-1</sup> whereas UHM-5 culture supernatants yielded 2 µg of glucose ml<sup>-1</sup>. The Exo<sup>-</sup> derivative and the parental strain gave essentially similar growth in medium containing from 0 to 300 µM Al, indicating that the pSym of CIAT899, and extracellular polysaccharide, were not involved in the aluminum tolerance of this strain. However, increasing the level of Al from 80 to 150 µM increased the lag phase, induced a slight killing of the inoculum, and depressed the final populations by about fivefold. Doubling the aluminum concentration from 150 to 300 µM presented a severe aluminum stress to CIAT899 and UHM-5: the inoculum level dropped 10-fold, indicating killing of the inoculum, and remained depressed for ca. 4 days before continuing to grow slowly; the final population was decreased 15-fold relative to that of cultures grown in medium containing 80 µM Al. The production by CIAT899 of other extracellular or intracellular aluminum tolerance factors was investigated in culture by using aluminum-sensitive rhizobia as stress indicators. These experiments, conducted at 80 µM Al, demonstrated that CIAT899 produced neither extracellular nor intracellular products that could alleviate toxicity for the Al-sensitive indicator rhizobia.**

The adverse effects of aluminum on a variety of biological systems, from bacteria to humans, are well documented (14, 20, 22, 25, 29, 35-37). Such reports imply that the biological effects attributed to aluminum occur over a variety of pH, phosphate, and other ion gradients. In agriculture, however, Al toxicity is usually a problem encountered only with acidic soils (pH ≤5). In the tropics, stressful soil conditions, such as high acidity and the presence of toxic levels of aluminum and manganese, are often encountered. Such soil conditions often lead to problems with the successful cultivation of legumes through a failure of the *Rhizobium* inoculant to either establish in the soil, compete successfully with indigenous rhizobia, or proceed through acid- and aluminum-sensitive steps in the infection process (17, 18).

The mechanism of aluminum resistance in *Rhizobium* spp. is not understood. It is a trait which varies markedly from strain to strain. It is known that aluminum-resistant strains are also resistant to acidity. They are independent properties, however, since acid tolerance does not guarantee aluminum tolerance (12, 18, 23, 24). For selecting strains that form effective symbioses in acid soils, screening of rhizobia in the laboratory for growth in acid culture media with and without aluminum has proven useful (18, 44, 47). Tolerance of this type appears to be stable, uninfluenced by previous culture conditions (31). Acid tolerance is positively corre-

lated with exopolysaccharide (EPS) production (10); however, the ability of EPS to chelate Al was not correlated with a strain's acid tolerance (11). Tremaine and Miller (44a) compared EPS production and Al binding of EPS extracted from seven strains of *Rhizobium leguminosarum* biovar phaseoli and *Bradyrhizobium japonicum* differing in resistance to Al. One Al-tolerant strain (*R. leguminosarum* bv. phaseoli C-12) produced significantly more EPS than other strains; this EPS complexed 10 to 100 times more aluminum than EPS from the other strains. Incorporation of C-12 EPS into the growth medium relieved Al toxicity for the Al-sensitive strains. The only EPS from Al-tolerant strains to function in this manner came from strain C-12; Tremaine and Miller therefore concluded that Al tolerance, in general, was not due to EPS chelation of the metal. The role of EPS in Al tolerance of *Rhizobium* spp. remains uncertain. Reports suggest that EPS may play a role in aluminum tolerance (2, 3); other recent investigations indicate that DNA may be the site of action of aluminum in *Rhizobium* spp. (22).

In this report, we describe investigations of possible mechanisms of aluminum resistance in *R. leguminosarum* bv. phaseoli CIAT899. The role of EPS was investigated through creation of a nonmucooid (Exo<sup>-</sup>) mutant. The potential for excreted or other cell surface factors was explored by "preconditioning" medium with wild-type CIAT899 and by coculturing wild-type CIAT899 with aluminum-sensitive indicator strains. Finally, the presence of an "internal factor(s)" was examined through the addition of a cell extract isolated from CIAT899 cells grown in the presence of aluminum to cultures of an indicator strain. The results demonstrated that EPS was not important, additional cell surface or excreted factors did not appear to be responsible, and no readily demonstrable internal factor(s) capable of rendering aluminum less toxic to indicator strains was identified.

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‡ While this manuscript was in preparation, Ben Bohlool was killed, tragically, in an automobile accident. His unique approach to microbial ecology brought great enjoyment to learning about and appreciating the tremendous diversity of our microbial world. His students and colleagues, the staff of the NiffAL Project, and especially his wife, Carol, and daughter, Kiaora, will miss his presence greatly.

TABLE 1. Bacterial strains and characteristics

Strain	Description	EPS production ( $\mu\text{g}$ of glucose/ $10^9$ cells)	Reference or source
<i>R. leguminosarum</i> bv. <i>leguminosarum</i> Pre 6015(pJB6JI)	Al <sup>s</sup> Contains plasmids of 100, 165, 195, 285, and 310 MDa	ND <sup>a</sup> ND	T. A. Lie 7, 21
<i>R. leguminosarum</i> bv. <i>phaseoli</i> CIAT899 UHM-5 TAL-1472	Wild type, Al <sup>r</sup> CIAT899 pSym <sup>-</sup> Exo <sup>-</sup> Wild-type, Al <sup>s</sup>	185 2 ND	D. Munns (18) This study P. Somasegaran
<i>Rhizobium</i> sp. strain USDA 3HOa9	Al <sup>s</sup>	ND	27

<sup>a</sup> ND, not determined.

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used in this study and their relevant characteristics are listed in Table 1. *Rhizobium* strains were maintained on yeast extract-mannitol (YEM) (6) agar slants, cultured at 28°C, and stored at 4°C. For long-term storage, either slants were overlaid with sterile mineral oil or thick suspensions of cells were lyophilized in 2x skim milk (Difco). PA medium (21) was used for curing strain CIAT899 of the symbiosis plasmid (40). Plasmid profiles were determined by gel electrophoresis (39); plasmid size was estimated by mobility relative to plasmids of *R. leguminosarum* bv. *phaseoli* 6015(pJB5JI) (7, 21, 40).

**Total counts and enumeration of viable cells.** Cultures were adjusted to the desired number of cells by using Petroff-Hauser total counts. The actual titers of the inocula were verified by counting viable cells on plates of YEM (6) containing 0.05% bromthymol blue, a pH indicator (YEMBTBplates) (26, 27, 45).

**Growth of rhizobia at low pH and in the presence of aluminum.** *Rhizobium* strains were grown in a modified medium of Graham et al. (18) (minimal aluminum medium [MAM]), in which glycerol was used the carbon source and which contained 69 mM glycerol (final concentration), 11 mM sodium glutamate (final concentration), 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 460  $\mu\text{M}$  CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.5 mM (1.12 g) KCl, 30  $\mu\text{M}$  Fe-EDTA, KH<sub>2</sub>PO<sub>4</sub> (5  $\mu\text{M}$  P), 0.9  $\mu\text{M}$  MnSO<sub>4</sub>·H<sub>2</sub>O, 0.4  $\mu\text{M}$  ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.1  $\mu\text{M}$  CUCl<sub>2</sub>·2H<sub>2</sub>O, 0.02  $\mu\text{M}$  Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 2.7  $\mu\text{M}$  Co(NO<sub>3</sub>)<sub>2</sub>·2H<sub>2</sub>O, and sodium pantothenate, thiamine·HCl, and niacin at 8  $\mu\text{g}$  ml<sup>-1</sup> each (final concentration). Aluminum stock solutions (10x) were freshly prepared by using AlCl<sub>3</sub>·6H<sub>2</sub>O dissolved in double-distilled water to provide 80, 150, 300, and 500  $\mu\text{M}$  Al (final concentration) and adjusted with concentrated HCl so that 1 part of the Al stock solution to 9 parts of sterile MAM (pH 6) produced the desired final pH. Aluminum and acid stock solutions were filter sterilized before use. For MAM-YX, the above medium was supplemented with 0.001% yeast extract before autoclaving; for plates, 0.7% agarose was added prior to autoclaving.

Inoculum cultures were grown for 24 to 36 h in MAM (pH 6, 0  $\mu\text{M}$  Al) before being titered. All experiments consisted of three replicate flasks per treatment; the results are plotted as means of the replicates, and error bars indicate standard errors of the mean.

**Measurement of aluminum in media and solutions.** The concentration of aluminum in culture supernatants and solutions was determined by a modification of the colorimetric eriochrome cyanine R method (1); the volumes for all reagents and samples were reduced by a factor of 10, for a

working volume of 5 ml. For culture supernatants, cells were removed by centrifugation for 2 min in an Eppendorf micro-centrifuge (model 5412; Brinkmann Instruments, Inc., Westbury, N.Y.). The standard curve and sample analyses were performed in triplicate in disposable glass tubes (18 by 100 mm) and 4.5-ml disposable polystyrene square cuvettes. The A515 of the calibration curve and samples was measured on a Spectronic 710 spectrophotometer (Bausch & Lomb Inc., Rochester, N.Y.).

**Fluorescent-antibody techniques.** Fluorescent antibodies were prepared against the somatic components of *Rhizobium leguminosarum* bv. *phaseoli* CIAT899 and TAL-1472 and *Rhizobium* sp. (*Cicer arietinum*) strain USDA 3HOa9 by the method of Schmidt et al. (42) as described previously (27), except for the following modifications: equal numbers of organisms from early-log-, mid-log-, and late-log- or stationary-phase cultures were mixed together to provide the antigen for injection, and antibodies were conjugated in carbonate-bicarbonate buffer (pH 9) (46). Strain TAL-1472 failed to produce an adequate agglutination titer with the standard immunization procedure. A special procedure involving footpad injections of a dense antigen suspension ( $10^{11}$  to  $10^{12}$  cells ml<sup>-1</sup>) in complete Freund's adjuvant (1:1) boosted the agglutination titer sufficiently to allow preparation of a fluorescent antibody. Fluorescent antibody to *R. leguminosarum* strain Pre was prepared by B. Bohlool according to the protocol of Schmidt et al. (42).

Preparation of smears from broth cultures or nodules for immunofluorescence identification and immunofluorescence membrane filter counts were performed as described previously (26, 27). Immunofluorescence cross-adsorption (4), as described previously (27), was used to assess serological relatedness.

**EPS quantitation.** Analysis of total carbohydrate (glucose equivalents) of dialyzed culture supernatants of YEM brothgrown cells (CIAT899 and UHM-5) was performed by the anthrone method (19). Analysis of cultures grown in YEM broth (strains CIAT899 and UHM-5) was performed when the cultures reached a density of  $10^9$  cells ml<sup>-1</sup>, as determined by total counts (Petroff-Hauser counting chamber). All tests were run in triplicate. The cells were removed by centrifugation at 18,000 x g for 90 min. The supernatants were dialyzed exhaustively at 4°C against 0.85% saline. YEM broth was included as a control. All tests were run in triplicate.

**Extracellular products (preconditioned medium). Part 1.** *R. leguminosarum* bv. *phaseoli* CIAT899 (Al<sup>r</sup>) was inoculated to give  $10^6$  cells ml<sup>-1</sup> (viable-cell count) into 500 ml of MAM-YX-Al (80  $\mu\text{M}$  Al, pH 4.7) and 500 ml of MAM

YX-H+ (0  $\mu$ M Al, pH 4.7) media. Strains Pre (AP) and USDA 3HOa9 (Al<sup>s</sup>) were each inoculated to give  $10^6$  cells ml<sup>-1</sup> into flasks containing 200 ml of MAM-YX-Al (80  $\mu$ M Al, pH 4.7) and MAM-YX-H+ (0  $\mu$ M Al, pH 4.7) media. The flasks were incubated for 3 days at 28°C on a rotary shaker. Growth was estimated visually by the turbidity of the medium.

**Part 2.** After the initial 3-day growth period, the cells were removed from the medium by centrifugation (10,000 x g), and the level of aluminum and the pH of the spent media were measured and readjusted as necessary. The lots of spent media conditioned by the strains in part 1 were then sterilized in bulk by filtration through 0.45- $\mu$ m-pore-size nitrocellulose filters. The two sets of media (acid control and 80  $\mu$ M Al), preconditioned by CIAT899, were each aseptically dispensed in 50-ml aliquots into nine sterile 125-ml Erlenmeyer flasks. Triplicate sets of flasks were inoculated with (i) CIAT899 to give  $10^6$  viable cells ml<sup>-1</sup>, (ii) strain Pre to give  $10^6$  viable cells ml<sup>-1</sup>, and (iii) strain USDA 3HOa9 to give  $10^6$  cells ml<sup>-1</sup>. The two sets of media (acid control and 80  $\mu$ M Al) preconditioned by strains Pre and USDA 3HOa9 were each aseptically dispensed in 50-ml aliquots into three sterile 125-ml Erlenmeyer flasks. The sets of flasks for each strain-medium combination were inoculated with strain CIAT899 to give  $10^6$  viable cells ml<sup>-1</sup>. All flasks were placed on a rotary shaker for 3 days at 28°C. Growth was determined visually by the turbidity of the flasks relative to that of barium sulfate turbidity standards (34).

**Coculture.** A set of five strain treatments were designed to determine whether coculture of an aluminum-resistant strain (CIAT899) growing together in the same flask with an aluminum-sensitive strain (Pre or USDA 3HOa9) could alleviate the toxic effects of aluminum for the sensitive strains; strains grown individually served as controls. Each treatment set consisted of three flasks each containing 50 ml of MAM-YX-H<sup>+</sup> (pH 4.7) and three flasks each containing 50 ml of MAM-YX-Al (80  $\mu$ M Al, pH 4.7). All strains were inoculated to give an initial viable-cell count of about  $10^5$  cells ml<sup>-1</sup>. The growth of individual strains (CIAT899, Pre, and USDA 3HOa9) and treatments involving paired combinations of an aluminum-resistant strain with an aluminum-sensitive strain (i.e., CIAT899 with Pre and CIAT899 with USDA 3HOa9) was monitored for 4 days by immunofluorescence membrane filter microscopy.

**CIAT899 cell extract.** A cell extract was made from *R. leguminosarum* bv. phaseoli CIAT899 grown in 6 liters of MAM-Al (500  $\mu$ M Al, pH 4.45) for 10 days. The cells were harvested by centrifugation (10,000 x g), and the pellets were resuspended in pH 3 MAM salts (without Al) and combined. The combined pellet was washed three times in pH 3 MAM salts. The final yield was 2.1 g of cells (wet weight). The pellet was resuspended in 25 ml of 25 mM Tris-HCl (pH 6) and passed three times through a French pressure cell (Aminco model J4-3339; American Instrument Co., Silver Spring, Md.) at 35,000 lb/in<sup>2</sup> at 4°C (approximately 74% cell breakage). The protein concentration of the extract was determined by the method of Hansen and Phillips (19) and adjusted with 25 mM Tris-HCl (pH 6) to provide a protein content equivalent to  $10^{10}$  CIAT899 CFU ml<sup>-1</sup> when 0.5 ml was added to 50 ml of MAM (i.e., 1% extract). The extract was filtered through 0.8- $\mu$ m nitrocellulose filters and then sterilized through sterile 0.45- $\mu$ m nitrocellulose filters and frozen at -20°C. Aliquots of this extract (0.5 ml) were added to 50 ml of MAM (pH 4.45) with and without 80  $\mu$ M aluminum. There were three replicate flasks per treatment and a total of eight treatments (i.e., two

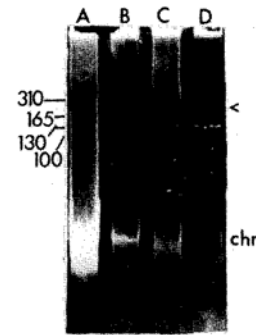


FIG. 1. Plasmid profiles of wild-type and pSym-cured *Rhizobium* strains. Lanes: A, 6015(pJB5JI); B, TAL-1472; C, UHM-5; D, CIAT899. Arrow opposite lane D indicates the ca. 220-MDa pSym plasmid of strain CIAT899, which was cured from strain UHM-5 (C); chr indicates chromosomal DNA. The numbers in the left margin refer to the sizes of the plasmids in megadaltons. Note: the two largest plasmids of strain 6015(pJB5JI) (285 and 310 MDa [7]) are not readily visible in this preparation of 6015(pJB5JI).

strains, TAL-1472 [Al<sup>r</sup>] and CIAT899 [Al<sup>r</sup>], in media with and without aluminum and with or without extract). The strains, grown in MAM at pH 6, were inoculated into the various treatment flasks to yield ca.  $10^5$  cells ml<sup>-1</sup>. Growth of the strains was monitored by viable-cell counts.

**Nodulation tests.** Bean seeds (*Phaseolus vulgaris* cv. Hawaiian Wonder) (University of Hawaii Department of Horticulture) were briefly rinsed with 95% ethanol and then surface sterilized in a solution of 3% hydrogen peroxide for 10 min. The seeds were rinsed three times with sterile distilled water and allowed to imbibe sterile distilled water for 1 h. Three seeds were aseptically transferred to plant growth jars (40); the jars were placed in a 28°C incubator and allowed to germinate for 3 days. Following germination, the seedlings were thinned to one per jar and inoculated with 1 ml of a log-phase culture containing  $10^8$  to  $10^9$  cells ml<sup>-1</sup>. The surface of the vermiculite-perlite mixture was topped with 3 to 5 cm of silicone-coated sand (5 kg of sand per 100 ml of silicone sealant; Bondex International, Inc., St. Louis, Mo.) to prevent cross-contamination. Plant tests were done in triplicate.

## RESULTS

**Role of EPS in aluminum tolerance.** Strain UHM-5 is an Exo<sup>-</sup> derivative of CIAT899. This variant was created during attempts to cure CIAT899 of plasmid pSYM. We were unable to cure CIAT899 by either standard acridine orange (up to 20  $\mu$ g ml<sup>-1</sup>) (40, 48) or heat (up to 40°C) treatment (40, 49). However, growth in PA medium (21) supplemented with 20  $\mu$ g of acridine orange ml<sup>-1</sup> at 37°C cured CIAT899 of its largest plasmid (ca. 220 MDa), leaving a smaller cryptic plasmid (Fig. 1, lanes C and D). These derivatives, missing the largest plasmid, were unable to nodulate their host, *P. vulgaris* cv. Hawaiian Wonder.

All colonies which resulted from this plasmid-curing treatment appeared to be affected in EPS production. One such pSym<sup>-</sup> (Nod<sup>-</sup>) Exo<sup>-</sup> derivative was designated UHM-5. UHM-5 gave a 4+ reaction with CIAT899 fluorescent antibody (i.e., homologous reaction). As determined by immunofluorescence cross-adsorption (4, 27), UHM-5 was indistinguishable antigenically from its gummy parent. Wild-type CIAT899 colonies were extremely gummy and spreading.

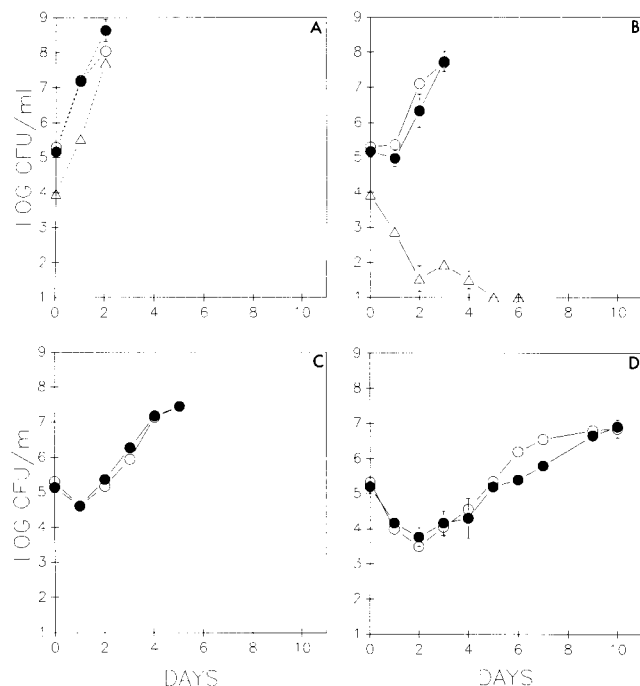


FIG. 2. Growth of wild-type CIAT899 and Exo<sup>-</sup> derivative UHM-5 in four levels of aluminum. Strains CIAT899 (●), mutant UHM-5 (○), and aluminum-sensitive indicator strain TAL-1472 (△) were tested in (A) 0  $\mu$ M Al, (B) 80  $\mu$ M Al, (C) 150  $\mu$ M Al, and (D) 300  $\mu$ M Al. (TAL-1472 used in the 0 and 80  $\mu$ M Al treatments only.)

The acidic extracellular polysaccharide of the wild-type strain caused the pH of YEM-BTB plates to decrease (turn yellow) after 3 days. By contrast, colonies of UHM-5 appeared to be "rough" on YEM-BTB plates; the colonies were pinpoint and yellow (acidic), while the medium remained green (neutral). Unlike with the wild-type parental culture, the pH of the plate did not decrease even after prolonged incubations (up to 2 weeks). CIAT899 produced smooth convex colonies on MAM agar plates at pH 6 and 4.45 (with and without 80  $\mu$ M Al). UHM-5 produced small pinpoint colonies on the same media. Agarose was used to solidify this medium; with regular agar, it was difficult to achieve the desired (final) acidic pH (i.e., the agar acted as a buffer), and the agar also appeared to chelate Al (Al-sensitive strains grew on plates supposedly containing greater amounts of Al than they could tolerate in broth). The use of agarose eliminated these problems.

The quantity of EPS contained in culture supernatants of CIAT899 and UHM-5 ( $10^9$  cells ml<sup>-1</sup>) grown in YEMS broth is listed in Table 1. UHM-5 produced approximately 1% of the parental level of EPS. The inability to produce EPS did not affect the growth of strain UHM-5 in the presence of 0, 80, 150, or 300  $\mu$ M Al (pH 4.45) relative to the growth of parental CIAT899 (Fig. 2). TAL-1472 was included as a negative control only in the acid and 80  $\mu$ M Al treatments. Growth was assayed by viable-cell counts. The addition of 80  $\mu$ M Al to the medium induced a 1-day lag in the growth of CIAT899 and UHM-5 (Fig. 1B). Al at 80  $\mu$ M proved lethal to strain TAL-1472, which died out in approximately 2 days. Increasing the level of aluminum in the medium to 150  $\mu$ M (Fig. 2C) or to 300  $\mu$ M (Fig. 2D) extended the lag phase and depressed both the growth rate and the maximum titer. Al at 300  $\mu$ M proved toxic to the inoculum, decreasing the initial

population 10-fold, indicating that only ca. 10% of the starting inoculum survived the initial exposure to 300  $\mu$ M Al. After 4 days, the strains recovered and grew slowly (Fig. 2D).

**Extracellular products (preconditioned medium).** The rationale for this experiment was to determine whether strain CIAT899, when grown in aluminum-containing media, produced a factor which would render aluminum-containing media less toxic to aluminum-sensitive indicator *Rhizobium* strains.

The results indicated that strains Pre and USDA 3HOa9 grew readily in acid media (pH 4.7) lacking aluminum. (These strains required the addition of 0.001% yeast extract to grow in the minimal medium and were not as acid tolerant as strain TAL-1472.) Strains Pre and USDA 3HOa9 failed to grow in media containing 80  $\mu$ M aluminum. In contrast, the aluminum-resistant strain CIAT899 grew well under both conditions. The results of part 2 of the experiment indicated that CIAT899 apparently did not excrete into the medium containing aluminum any factor that was able to counteract the toxic effects of aluminum on either of the sensitive strains Pre and USDA 3HOa9—these strains were unable to grow in MAM-YX-Al preconditioned by strain CIAT899. Strains Pre and USDA 3HOa9 grew readily in the preconditioned medium lacking aluminum (MAM-YX-H<sup>+</sup>). Therefore, the absence of growth in the aluminum-containing medium was due to aluminum and not to the production of some toxic factor by CIAT899.

Since the level of aluminum was readjusted to 80  $\mu$ M prior to reinoculation for part 2, any excreted product produced by CIAT899 might have been overwhelmed. To examine the latter possibility as well as to determine whether CIAT899 could alleviate the toxic effects of aluminum enough to allow a sensitive strain to grow when cocultured with it, the next experiment was performed.

**Coculture.** Figure 3A illustrates the growth of strains CIAT899, Pre, and USDA 3HOa9 in MAM-YX-AL (80  $\mu$ M Al, pH 4.7) and MAM-YX-H<sup>+</sup> (pH 4.7), as determined by membrane filter immunofluorescence counts (27); the strains were grown separately. All strains grew readily in acid media lacking aluminum, although CIAT899 grew more readily at this acidic pH. In media containing 80  $\mu$ M aluminum, only CIAT899 grew. Because of the nature of immunofluorescence filter counts, cell death (unless accompanied by cell lysis) would not be observed. Figures 3B and C illustrate the results of the Coculture experiments in which mixed populations (CIAT899 plus USDA 3HOa9 and CIAT899 plus Pre) in the same flask were monitored by membrane filter immunofluorescence microscopy (27). In Coculture, the paired strains grew under acid conditions (0  $\mu$ M Al, pH 4.7); however, in media containing 80  $\mu$ M aluminum, of the pair of strains within the same flask, only aluminum-tolerant strain CIAT899 grew. There was no increase in the population of sensitive strains above the level at which they were inoculated.

**CIAT899 cell extract.** Since nothing released by CIAT899 into the growth medium (1) or potentially involved with actively growing cells (2) could alleviate aluminum toxicity for the Al-sensitive indicator strains, an internal component(s) might be involved in the aluminum resistance of CIAT899. Such a component, if isolated and added to the growth medium, could potentially eliminate aluminum toxicity for the sensitive strain *R. leguminosarum* bv. phaseoli TAL-1472.

Aliquots of CIAT899 cell extract, produced from cells grown in MAM containing 500  $\mu$ M aluminum, were added to

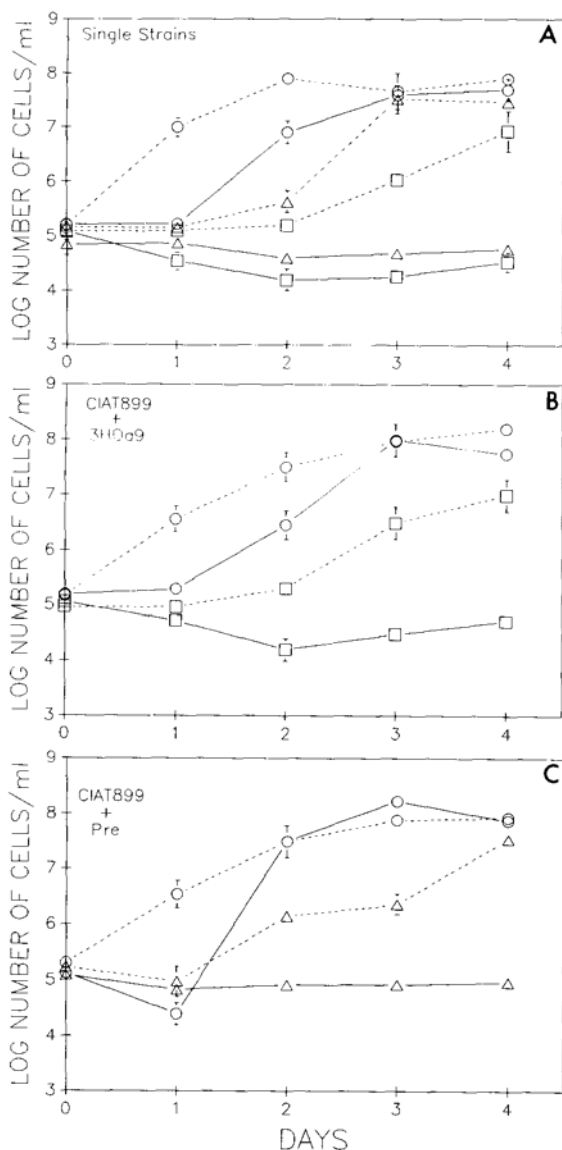


FIG. 3. Coculture: growth of aluminum-tolerant CIAT899 together with an aluminum-sensitive strain. Growth was monitored by quantitative membrane filter immunofluorescence microscopy. Key: ---, pH 4.7, 0  $\mu\text{M}$  Al; —, pH 4.7, 80  $\mu\text{M}$  Al. Strains CIAT899 (O), USDA 3HOa9 ( $\square$ ), Pre ( $\Delta$ ). (A) Single-strain (axenic) controls. (B) CIAT899 plus USDA3HOa9 cocultured simultaneously in same flask. (C) CIAT899 plus Pre cocultured simultaneously.

50 ml of MAM with and without aluminum to provide a protein content equivalent to  $10^{10}$  CIAT899 CFU  $\text{ml}^{-1}$ . This protein level represented an approximately 100-fold increase over the maximum titer of CIAT899 attained in MAM containing 80  $\mu\text{M}$  Al (i.e., ca.  $10^8$  CFU  $\text{ml}^{-1}$ ). The growth of strains CIAT899 and TAL-1472 was monitored for 7 days by viable-cell counts and is illustrated in Fig. 4.

Although the addition of CIAT899 cell extract did not affect the growth of strain CIAT899 or TAL-1472 in medium without aluminum (MAM- $\text{H}^+$ , pH 4.45), addition of the extract to flasks with 80  $\mu\text{M}$  aluminum led to a more rapid rate of cell death for strain TAL-1472 compared with the treatment without extract (Fig. 4). The cell extract had no protective effect.

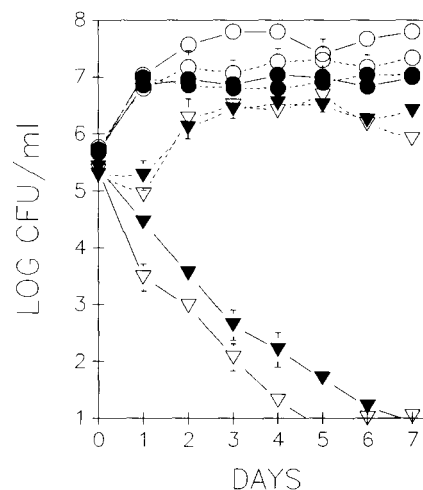


FIG. 4. Effect of incorporation of CIAT899 cell extract on the growth of CIAT899 and TAL-1472 in 0 and 80  $\mu\text{M}$  Al. Symbols: O, ●, CIAT899; ▽, ▼, TAL-1472; ●, ▼, no extract; O, ▽, 1% extract (1%, vol/vol; protein equivalent to  $10^{10}$  CIAT899 CFU  $\text{ml}^{-1}$ ); ---, 0  $\mu\text{M}$  Al; —, 80  $\mu\text{M}$  Al.

## DISCUSSION

Aluminum toxicity in agriculture is considered a problem only under acidic soil conditions ( $\leq \text{pH}$  4.5), when free aluminum ion ( $\text{Al}^{3+}$ ) increases rapidly in solubility (30, 41). Although the solubility of soil aluminum (as  $\text{Al}^{3+}$ ) increases greatly under acidic soil conditions, the toxic effects of aluminum on biological systems appear to occur over a broad range of pHs, and this implies that different species of aluminum ion (i.e., aluminum ion complexes) invoke toxicity intracellularly; aluminum will interact with and bind to many cellular components (20, 25, 28, 29, 35, 37). The DNA of *Rhizobium* strains may be a possible site of action of aluminum (22). The solution chemistry of aluminum is a factor which greatly complicates the interpretation of data on the biological mechanism(s) of aluminum toxicity (28, 29).

Bacterial EPSs are known to bind and concentrate metal ions (8) and can thereby play a role in ameliorating metal toxicity (5, 13). *Rhizobium* EPSs also bind cations (43). Cunningham and Munns (10, 11) reported that pH tolerance in *Rhizobium* spp. was not correlated with the Al-binding capacity of EPS. In an unpublished report, Tremaine and Miller (44a) concluded that EPS binding of Al was not responsible for Al tolerance. However, Appanna (2, 3), who found both qualitative and quantitative differences in the EPS of *Rhizobium* strains grown in media containing aluminum, suggests that EPS may play a role in Al protection via binding. In this report, we demonstrated directly in the same genetic background that the lack of EPS (ca. 1% of the wild-type level) in Al-resistant CIAT899 had no effect on the growth of Exo<sup>-</sup> strain UHM-5 in levels of aluminum up to and including 300  $\mu\text{M}$ . Bacterial plasmids often encode resistance to toxic metal ions (13); however, there were no determinants of Al resistance on the pSym plasmid of CIAT899, since the loss of pSym did not affect either pH or Al tolerance, as has been noted by others (9, 16, 38). Since UHM-5 lacked the CIAT899 pSym and was Nod<sup>-</sup> in plant tests, the role of the EPS in preventing a chlorosis-inducing factor (32) was not observed. Although CIAT899 and UHM-5 behaved similarly at all Al levels tested, increasing the concentration of Al from 80 to 300  $\mu\text{M}$  proved a severe

stress to these strains. *R. trifolii* ANU1173 is tolerant of  $\leq 25 \mu\text{M}$  Al (38); increasing the concentration of aluminum above  $25 \mu\text{M}$  to  $50$  or  $80 \mu\text{M}$  led to cell death (27a). CIAT899, by contrast, was tolerant of an amount of aluminum at least five times greater. Due to this intrinsic high-level tolerance to aluminum, the experiments examining various possible aluminum tolerance mechanisms were conducted at  $80 \mu\text{M}$  Al on the assumption that the mechanism involved was efficient.

The aluminum tolerance mechanism of CIAT899 did not appear to involve an exported product(s), since preconditioning media with CIAT899 did not provide protection for aluminum-sensitive indicator strains. As well, there was no co-protective effect when CIAT899 was cultured along with aluminum-sensitive strains. The latter experiment eliminated any bias introduced in the preconditioning experiment when the level of aluminum was readjusted prior to re-inoculation. The incorporation of Al-grown CIAT899 cell extract into MAM broth did not provide protection from the toxic effects of aluminum when added to flasks containing an Al-sensitive strain. The extract was incorporated into the medium to provide a protein content of  $10^{10}$  CIAT899 CFU  $\text{ml}^{-1}$ . This represented a 100-fold increase in the equivalent titer of CIAT899 attained in media containing  $80 \mu\text{M}$  Al. Aluminum can enter and bind to the DNA of both tolerant and sensitive *Rhizobium* strains, leading to increased DNA synthesis in the sensitive strains (22). If interference with DNA synthesis is the mechanism of action, the experiments described here would not have detected that. Perhaps aluminum-sensitive organisms accumulate more aluminum than tolerant organisms, and this accumulated aluminum interferes with cellular processes (35-37). An exclusion method of Al tolerance by CIAT899 was not examined in this study.

The utilization of bacterial mutants affected in aluminum tolerance would enable more rapid progress in understanding the mechanism of aluminum resistance. Transposon Tn5 mutants of strain CIAT899 (16) and a strain of *R. meliloti* (33) affected in pH tolerance have been generated. Since pH mutants are rendered acid sensitive, resistance to aluminum as experienced under agricultural conditions cannot be evaluated. Acid-tolerant but aluminum-sensitive mutants have not yet been reported. An alternative approach to the creation of Al<sup>s</sup> mutants would be to determine whether cosmid clones from aluminum-tolerant strains impart tolerance when transferred to acid-tolerant, aluminum-sensitive recipients. Aluminum tolerance may be the outcome of overall genotypic fitness rather than one specific genetic determinant readily amenable to mutational analyses.

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